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THE DEVELOPMENT OF TOLERANCE TO ANTILIPOLYTIC AGENTS IN RATS

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Abstract—The development of tolerance to the action of certain antilipolytic agents has been investigated *in vivo* in rats. Tolerance to oral nicotinic acid developed during twice daily dosing for 4 days at 100 and 250 mg/kg but not at 10, 25 or 50 mg/kg. Tolerance induced by high doses of nicotinic acid was no longer detectable after a further week without treatment. Tolerance developed to a dose of 10 mg/kg nicotinic acid when dosing was repeated at hourly intervals for up to 6 hr. Rats made tolerant to nicotinic acid also became tolerant to both 5-methylpyrazole-3-carboxylic acid and to pyridyl-3-tetrazole and rats made tolerant to these antilipolytic agents were also tolerant to nicotinic acid. Rats made tolerant to nicotinic acid still responded to the antilipolytic activity of the prostaglandin analogue, sulprostone. These results suggest that nicotinic acid, pyridyl-3-tetrazole and 5-methylpyrazole-3-carboxylic acid act through a common mechanism or receptor and that the development of tolerance is associated with this receptor or the mechanism by which it is linked to adenylate cyclase.

Inhibition of lipolysis *in vivo* has been shown to reduce the concentrations of both blood glucose [1] and plasma triglycerides [2] and thus may offer therapeutic benefits in diabetes mellitus [3]. However, tolerance develops to the antilipolytic action of pyrazole and isoxazole antilipolytics in rats [4, 6] and in man [7, 8] and this has been considered to limit their therapeutic usefulness. In man nicotinic acid remains an effective antilipolytic and hypolipidaemic after prolonged treatment [7, 9]. Reports have suggested that in rats tolerance to nicotinic acid does not develop [5, 6] although pretreatment with 3-methylisoxazole-5-carboxylic acid causes cross tolerance to nicotinic acid [6]. Conflicting reports have been published as to the development of tolerance to the nicotinic acid analogue pyridyl-3-tetrazole [5, 10]. We have investigated more thoroughly the development of tolerance to nicotinic acid in rats.

MATERIALS AND METHODS

Animals. Unless otherwise stated all studies were carried out with male or female (180–220 g) Wistar–Sprague–Dawley rats cross-bred at Glaxo Group Research Ltd. (Ware, U.K.). Rats were maintained on standard rodent diet, B.P. No. 1 (B.P. Nutrition U.K. Ltd., Witham, Essex).

Materials. Nicotinic acid was obtained from Sigma Chemical Co. (London, U.K.). 5-methylpyrazole-3-carboxylic acid, pyridyl-3-tetrazole and sulprostone were synthesised by the Chemistry Research Department of Glaxo Group Research Ltd. (Ware, U.K.).

EXPERIMENTAL PROCEDURES

(a) *Tolerance studies.* For each experiment 36 rats were randomly subdivided into two groups of 18, one of which was orally dosed with vehicle (1% carboxymethylcellulose, 1 ml/kg body wt) and one with the compound under test. Rats were dosed twice daily (0830 and 1730 hr) for 4 days (pre-treatment doses) and were then fasted overnight. On day 5, each group was further subdivided into two groups: one (12 rats) was dosed with vehicle, whilst the other (6 rats) was dosed with test compound (challenge doses). Blood samples were collected immediately before this final dose and at selected time points afterwards. Blood samples were obtained by gentle massage of the tail of the unrestrained rat after the tip had been amputated. 50 μl blood samples were mixed with 50 μl 5% (w/v) EDTA made up in 0.9% (w/v) NaCl and centrifuged: the supernatants were assayed for non-esterified fatty acids (NEFA) by the method of Shimuzu *et al.* [11], in which AMP, liberated by the enzymatic conversion of fatty acids to their fatty acyl CoA derivative, is assayed conventionally using myokinase.

(b) *Cross tolerance studies.* 72 rats were used in each of these studies and were randomly subdivided into three groups of 24, one of which was orally dosed with vehicle, one with compound A and the third with compound B. Rats were dosed twice daily (0830 and 1730 hr) for 4 days and were then fasted overnight. On day 5, each pre-treatment group was further subdivided into a group of 12 rats which was dosed with vehicle, and two groups of 6 animals, one of which was dosed with compound A and the other with compound B. Blood samples were collected as described above.

Statistical analysis

Statistical significance was determined by analysis of variance. Two comparisons were made for each experiment: (i) % reductions in plasma (NEFA) caused by the antilipolytic agents were compared with control concentrations for each pre-treatment group at each time point after the challenge treatments. Statistical significance for this analysis is indi-

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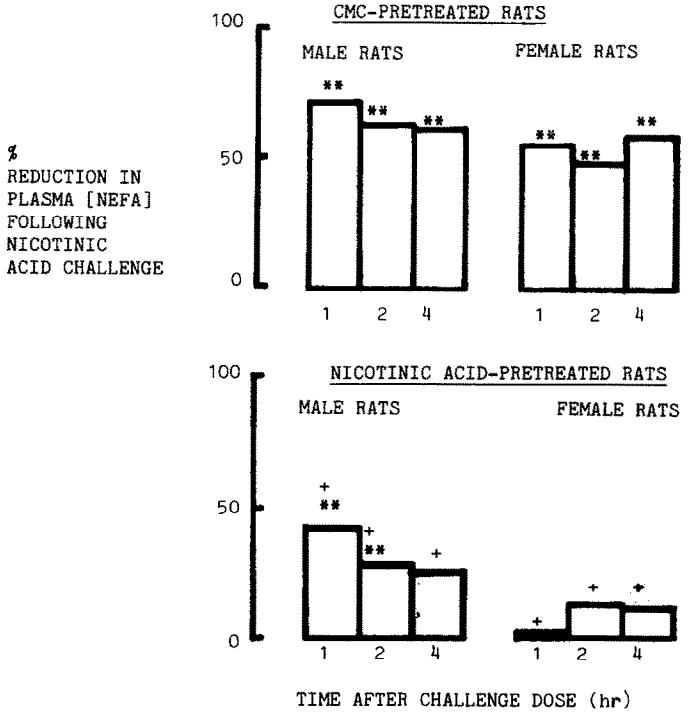

Fig. 1. Comparison of the development of tolerance to the antilipolytic action of nicotinic acid (250 mg/kg) in male and female rats. Rats were pretreated and challenged with nicotinic acid (250 mg/kg) as indicated in Methods. Control plasma [NEFA] (mean ± S.E.M.) at 0 hr were as follows: male CMC-pretreated rats 0.79 ± 0.07 mM; male nicotinic acid-pretreatment rats 0.97 ± 0.05 mM; female CMC-pretreated rats 0.74 ± 0.05 mM; female nicotinic acid pretreated rats 0.86 ± 0.05 mM.

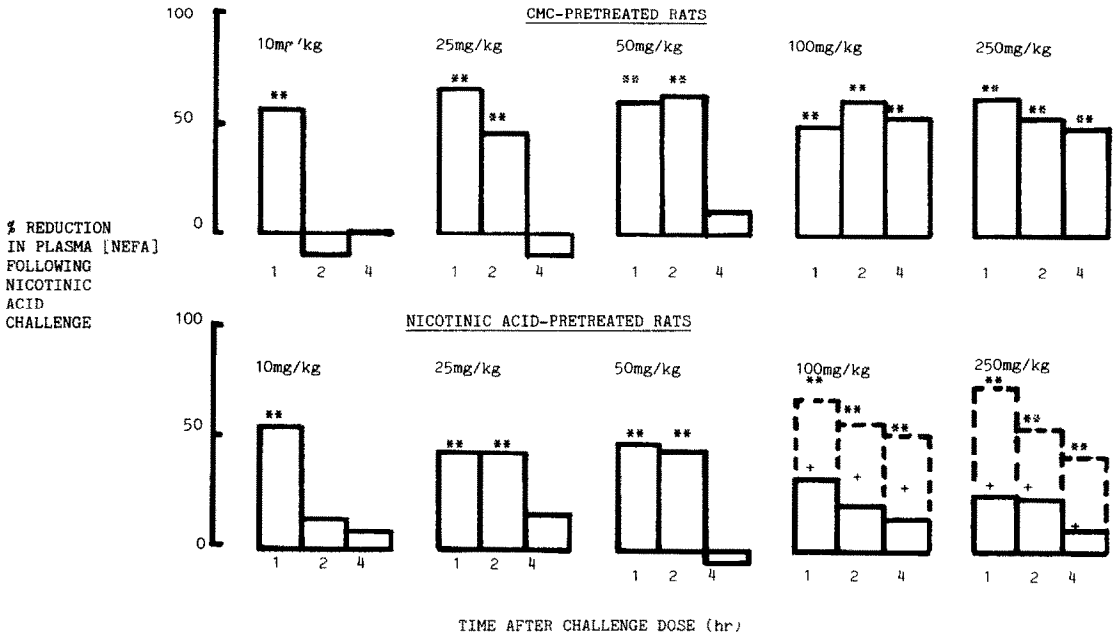

Fig. 2. The development of tolerance to the antilipolytic action of nicotinic acid at different doses. Groups of female rats were pretreated and challenged with the same dose of nicotinic acid as indicated in the figure, according to the protocol outlined in Methods. Control plasma [NEFA] (mean ± S.E.M.) at 0 hr for CMC-pretreated rats were as follows: for rats challenged with nicotinic acid at 10 mg/kg 1.02 ± 0.06 mM, 25 mg/kg 1.02 ± 0.06 mM; 50 mg/kg 1.02 ± 0.06 mM; 100 mg/kg 0.98 ± 0.05 mM; 250 mg/kg 0.98 ± 0.05 mM, nicotinic acid-pretreated rats were as follows: for rats challenged with nicotinic acid at 10 mg/kg 1.12 ± 0.04 mM; 25 mg/kg 1.18 ± 0.06 mM; 50 mg/kg 0.95 ± 0.06 mM; 100 mg/kg 0.99 ± 0.04 mM; 250 mg/kg 0.96 ± 0.05 mM. The dotted bars show the effect of nicotinic acid, given five days after the challenge dose during which no further treatment was administered, in groups pretreated and challenged with nicotinic acid at 100 and 250 mg/kg. (The results for the effect of nicotinic acid in CMC-pretreated rats challenged with nicotinic acid at 100 and 250 mg/kg and then kept for a further five days without further treatment are not shown as they are very similar to the results illustrated for the nicotinic acid challenge given after 4 days' pretreatment with CMC).

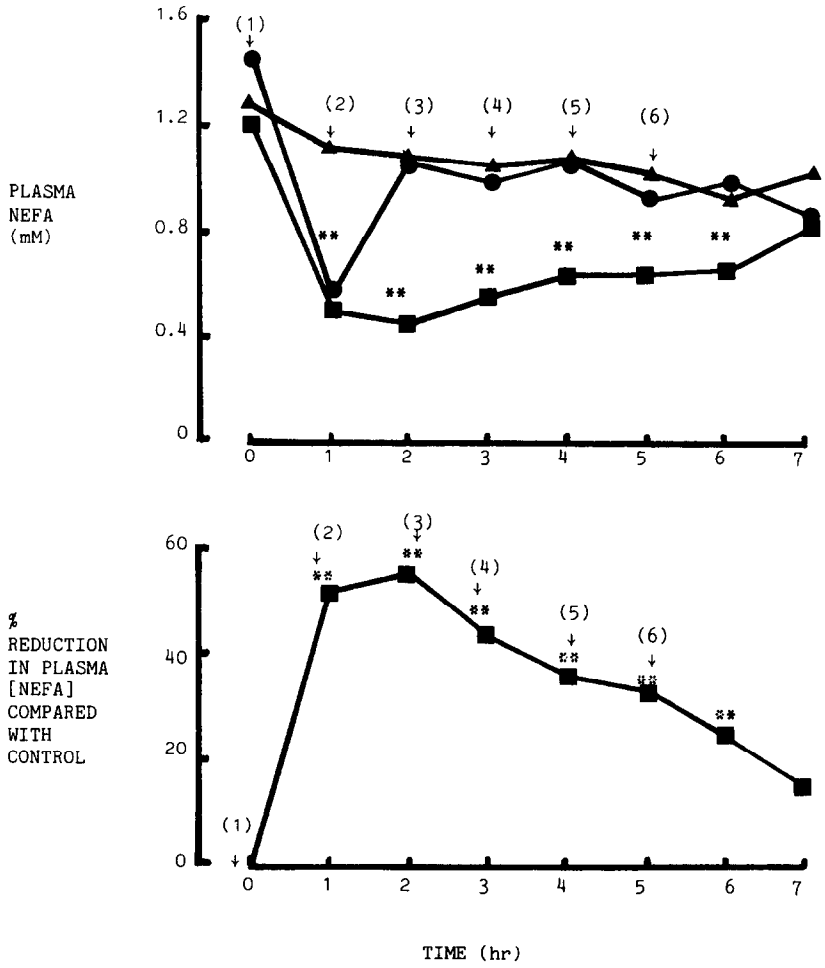

Fig. 3. The effect of repeated dosing of rats with nicotinic acid (10 mg/kg) on plasma [NEFA]. Three groups of female rats were starved overnight and then dosed on six occasions at 1-hr intervals (indicated by ↓) as follows: group 1 (12 rats) CMC dosed at (1)–(6) [▲]; group 2 (6 rats) nicotinic acid (10 mg/kg) dosed at (1) and then CMC dosed at (2)–(6) [●]; and group 3 (6 rats) nicotinic acid (10 mg/kg) dosed at (1)–(6) [■]. Panel (a) shows the mean [NEFA] over 7 hr for each group. Panel (b) shows the % fall in plasma [NEFA] for group 3 compared with group 1. Statistical significance of the % reduction in plasma [NEFA] in groups 2 and 3 compared with group 1: $^{**}P < 0.01$.

cated on the figures by $^{*}P < 0.05$ and $^{**}P < 0.01$ and (ii) the % reduction in plasma (NEFA) caused by the antilipolytic agents was compared in the different pre-treatment groups at each time point after the challenge treatment with that seen in the CMC-pretreated group. This analysis was carried out at the 95% confidence level and is indicated on the figures by $+P < 0.05$.

RESULTS

The development of tolerance to nicotinic acid in rats

Figure 1 shows that pretreatment of both male and female rats with 250 mg/kg nicotinic acid twice daily for four days reduced the ability of a subsequent dose to lower the plasma NEFA concentration on these rats. As the doses of nicotinic acid used in man (1.5 g, 21 mg/kg three times daily [7]) are much lower than this dose a range (10–250 mg/kg) of doses was examined for their ability to induce tolerance (Fig. 2). Tolerance developed after pretreatment with 100 or 250 mg/kg but not after 10, 25 or 50 mg/kg. When rats in which tolerance had developed were kept for a further five days after their challenge dose recovery from tolerance was complete (Fig. 2). In these experiments there was a sixteen hour interval between the final pretreatment dose and the challenge dose. As it was considered that tolerance to lower doses of nicotinic acid might have developed and then resolved during this time interval we investigated the effect of repeated dosing with 10 mg/kg at hourly intervals. A single dose of 10 mg/kg nicotinic acid had reduced plasma NEFA after one hour but a further hour later the NEFA concentration had returned to normal (Fig. 3a). Repeated dosing at hourly intervals for 6 hr caused a progressive decrease in the antilipolytic response (Fig. 3a, b).

Development of cross-tolerance between nicotinic acid and other antilipolytic agents

Figure 4 shows that pretreatment of rats with either nicotinic acid (250 mg/kg b.i.d.) or 5 methyl-

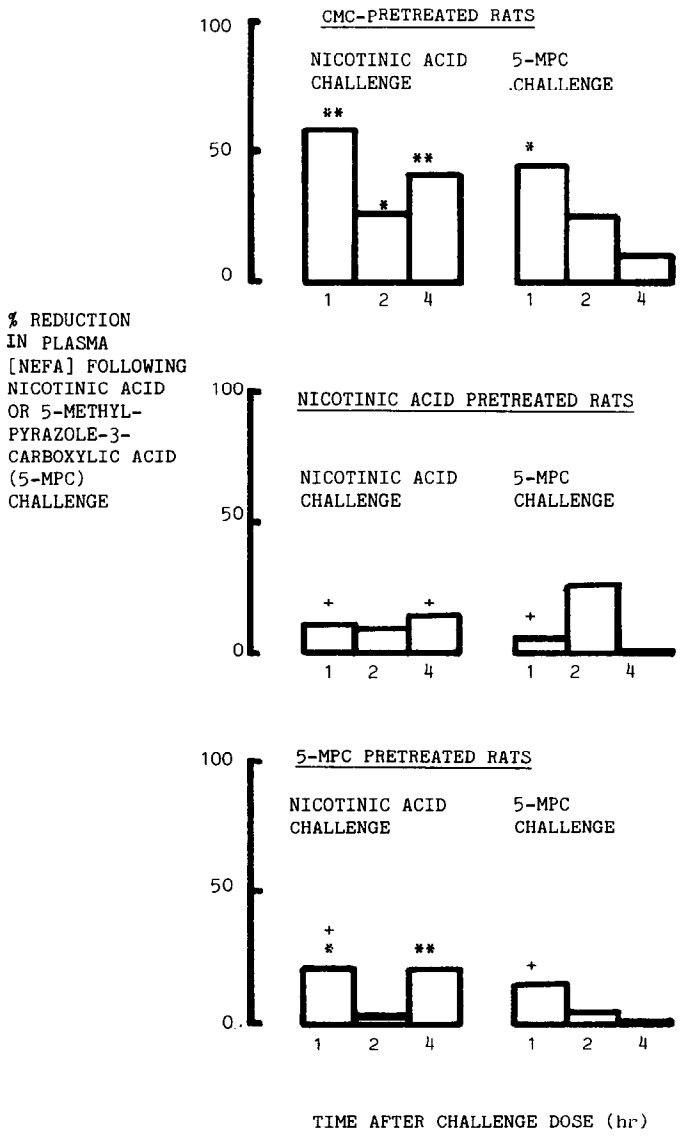

Fig. 4. The development of tolerance to and cross-tolerance between the antilipolytic actions of nicotinic acid (250 mg/kg) and 5-methylpyrazole-3-carboxylic acid (10 mg/kg) in female rats. Rats were pretreated and challenged with nicotinic acid (250 mg/kg) or 5-MPC (10 mg/kg) as described for cross-tolerance studies in Methods. Control plasma [NEFA] (mean ± S.E.M.) at 0 hr were as follows: CMC-pretreated rats 0.97 ± 0.06 mM; nicotinic acid-pretreated rats 0.95 ± 0.06 mM; 5-MPC pretreated rats 1.01 ± 0.05 mM.

pyrazole-3-carboxylic acid (10 mg/kg b.i.d.) for four days induced tolerance to both compounds. A similar cross tolerance developed between nicotinic acid and pyridyl-3-tetrazole (Fig. 5). In contrast, however, pretreatment with nicotinic acid failed to induce tolerance to the antilipolytic prostaglandin analogue, sulprostone (Fig. 6).

DISCUSSION

These results demonstrate unequivocally the development of tolerance to the antilipolytic activity of nicotinic acid in rats. These observations conflict with previous reports [5, 6] and these differences cannot be resolved on the basis of the sex of the animals used. Under the routine experimental conditions used (a 16 hr delay between the final pretreatment dose of nicotinic acid and the challenge dose), tolerance developed only after pretreatment with high doses of nicotinic acid. However, tolerance could be induced with an oral dose of 10 mg/kg if this dose was given at hourly intervals for 6 hr. Thus the development of tolerance is dependent both on the magnitude of the dose and on the frequency of dosing and may reflect the length of time for which the drug is present in the body at an effective concentration. The failure to detect the development of tolerance to nicotinic acid in man [7, 9] may be due to a combination of these factors. However, it should be noted that while a dose of 1.5 g nicotinic acid

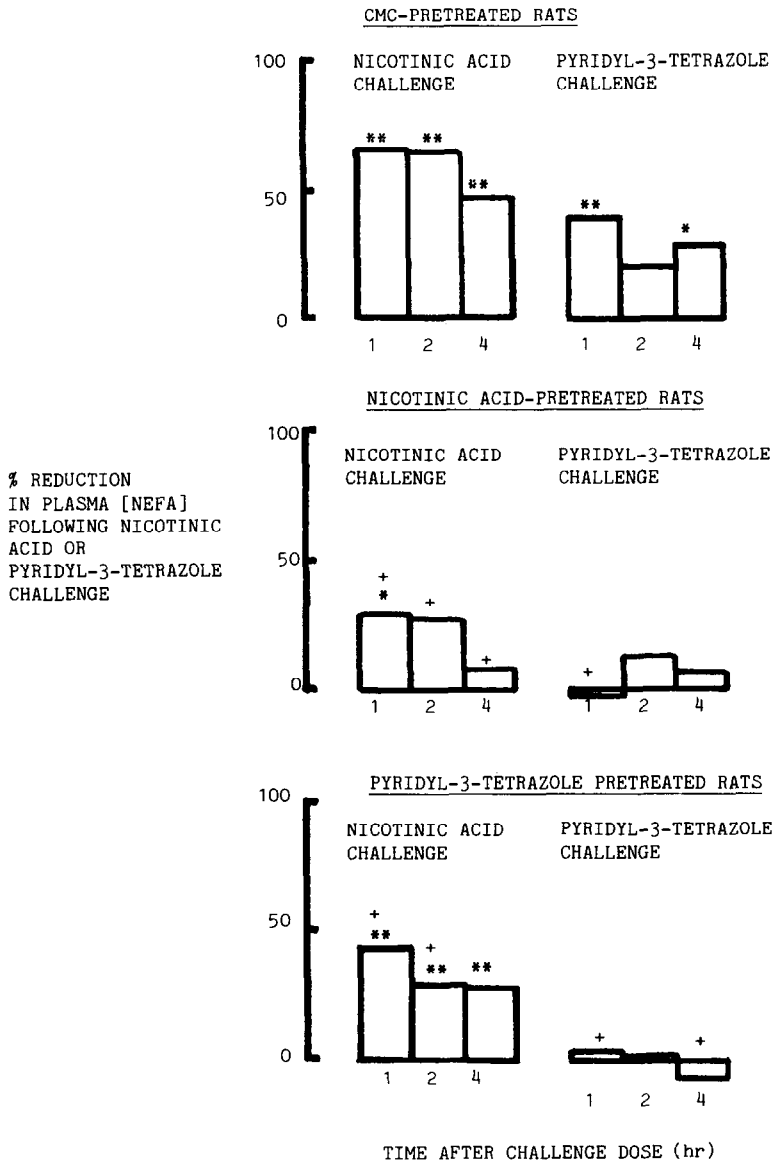

Fig. 5. The development of tolerance to and cross-tolerance between the antilipolytic actions of nicotinic acid (250 mg/kg) and pyridyl-3-tetrazole (200 mg/kg) in female rats. Rats were pretreated and challenged with nicotinic acid (250 mg/kg) or with pyridyl-3-tetrazole (200 mg/kg) as described for cross-tolerance studies in Methods. Control plasma [NEFA] (mean ± S.E.M.) at 0 hr were as follows: CMC-pretreated rats 1.14 ± 0.05 mM; nicotinic acid-pretreated rats 1.22 ± 0.05 mM; pyridyl-3-tetrazole-pretreated rats 1.15 ± 0.07 mM.

(equivalent to 21 mg/kg in a 70 kg subject) will reduce the concentration of NEFA in plasma for approximately 4 hr in man [7] a dose of 100 mg/kg is required to produce effects of the same duration in rats (Fig. 2).

The development of cross tolerance between nicotinic acid, 5 methylpyrazole-3-carboxylic acid and pyridyl-3-tetrazole indicates that these three antilipolytic agents share the same receptor and/or mechanism of action. Nicotinic acid and 5-methylpyrazole-3-carboxylic acid inhibit adenylate cyclase [12] and this has been claimed as the basis for their antilipolytic effect [13–16].

The prostaglandin E_2 analogue sulprostone is an inhibitor of adenylate cyclase and an antilipolytic agent in rat adipocytes [17, 18] exerting its activity via a specific receptor [17]. The failure of rats made tolerant to nicotinic acid to show tolerance to sulprostone indicates that tolerance to nicotinic acid does not develop at the level of adenylate cyclase but at some receptor or biochemical coupling mechanism prior to the cyclase. This site of action is common to that used by 5 methyl-pyrazole-3-carboxylic acid and pyridyl-3-tetrazole but is distinct from that employed by sulprostone. In addition we have demonstrated that tolerance to nicotinate can be induced *in vitro* using isolated adipocytes and these results are presented in the accompanying paper [19].

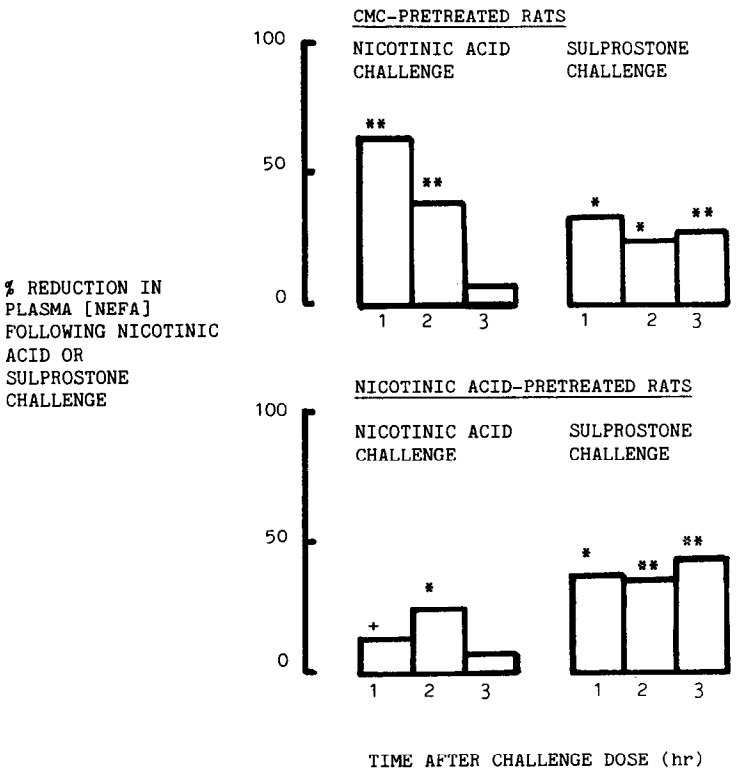

Fig. 6. Comparison of the development of tolerance to the antilipolytic actions of intravenously administered nicotinic acid (30 mg/kg) and sulprostone (0.5 mg/kg) in nicotinic acid pretreated rats (250 mg/kg, b.i.d., 4 days). Female rats were pretreated with CMC or nicotinic acid (250 mg/kg) twice daily for 4 days and then challenged with intravenously administered saline, nicotinic acid (30 mg/kg) or sulprostone (0.5 mg/kg) after an overnight fast. Control plasma [NEFA] (mean ± S.E.M.) at 0 hr were as follows: CMC-pretreated rats 1.11 ± 0.06 mM; nicotinic acid-pretreated rats 1.15 ± 0.08 mM.

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